## AMENDMENTS TO THE SPECIFICATION

In the Sequence Listing:

Applicants request consideration and entry of the Sequence Listing paper copy and computer readable copy. Pursuant to 37 C.F.R. 1.77, please enter the paper copy of the Sequence Listing after the Abstract.

Application No.: 10/690,949 3 Docket No.: 300622005001

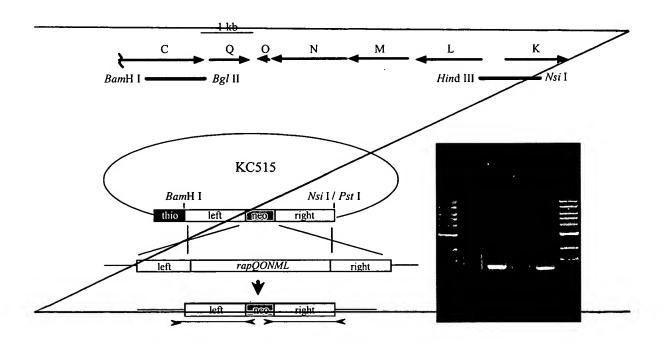
In the Specification:

Please amend the specification at page 5 by inserting the following new paragraph [0014.1] at line 12:

[0014.1]. Figure 2 shows the rapQONML region and the location of the two flanking sequences used in construction of recombinant phage KC515 (top); a diagram of the result of the double crossover event with the recombinant phage DNA (left bottom); and the results of the PCR reactions from wild-type and KOS20-001 strains (left right).

Please amend paragraph [0126] on page 39 as follows:

[0126] There remains a need for rapamycin analogues with improved activity and means for their production. The present invention meets such needs in that it provides, *inter alia*, a recombinant organism from which the five genes, rapQONML, have been replaced with a neomycin resistance marker and the compound 16-desmethyl-27-desmethoxyrapamycin, which cultures of this strain, when fed pipecolate, produce in significant amounts. The strain in which the rapQONML region was replaced with a neomycin resistance cassette was constructed as diagrammed below in Figure 2.



Please amend paragraph [0127] starting on page 39 as follows:

[0127] As shown above in Figure 2, sequences flanking the *rapQONML* region were cloned by PCR, the Tn5 neomycin resistance cassette of pFDNEO-S was inserted between them, and the cassette was ligated into the KC515 phage vector. The recombinant phage was used to obtain NeoR

lysogens of *S. hygroscopicus*, which were screened for the double crossover event by patching to agar medium containing both neomycin and thiostrepton. NeoR, ThioS strains were analyzed by PCR to confirm the presence of both amplimers spanning the homologous sequences. DNA from strain KOS20-001 gave both amplimers, indicating that the *rapQONML* genes had been replaced with the *neo* marker. Thus, in the above diagram, the *rapQONML* region and the location of the two flanking sequences used in the construction are shown. Also shown is the result of the double crossover event with the recombinant phage DNA; the location of the two amplimers used to verify the double crossover strain is indicated by lines between convergent arrows representing the primers. Finally, the results of the PCR reactions with DNA from wild type (lanes 1 & 3) and KOS20-001 (lanes 2 & 4) are shown. The primers amplified the left region (lanes 1 & 2) or the right region (lanes 3 & 4). The marker lanes (M) are New England Biolabs 1 kb ladder.

Please amend paragraph [0137] starting on page 44 as follows:

[0137] The cassette for replacement of *rapQONML* with a neomycin resistance gene was constructed as shown in above. Approximately 1.2 kb regions upstream and downstream of the *rapQONML* genes were obtained by PCR amplification from *S. hygroscopicus* genomic DNA. The location of each primer on the published rapamycin cluster sequence (1, 11, 15) given below refers to the 3' end of each primer. The upstream region was obtained with primers from nucleotide 89986

(5'-ATCGGATCCGC GCCAGGTCCGGCGACCCGTCCGCTTCC-3')
(SEQ ID NO: 1), introducing a BamH I site, and 91124

(5'-GATAGATCTAGACCGAAGGCCGACATCACGGTGTCGAAC-3')
(SEQ ID NO: 2), introducing a *Bgl* II site. The downstream region was obtained with primers from 95072

(5'-ATCAAGCTTG CTTGATGTCACGCTGGCACAGAACCTTGG-3')
(SEQ ID NO: 3), introducing a *Hind* III site, and 96218

(5'-GATATGCATCCGTGCCGTCCCAGGTTCTCGGCACCGATC-3') (SEQ ID NO: 4), introducing an *Nsi* I site. The PCR mixes included cloned *Pfu* polymerase (Stratagene), the manufacturer's buffer, 10% DMSO, 200 μM each of dATP, dTTP, dCTP, and 100 μM each of dGTP and deaza-dGTP (Roche). Thirty cycles of 30 sec at 95°, 30 sec at 60° and 3 min at 72° were used. Each amplimer was cloned into the *Srf* I site of pCR-Script (Stratagene) to give pKOS20-55.2 and pKOS20-56.1, respectively, and clones were verified by sequencing.

Please amend paragraph [0138] on page 45 as follows:

Plasmid pKOS7-150 was derived from Litmus 28 and has the following sequence between the *SnaB* I and *Avr* II sites: 5'-TGGATCCACAGATCTGCCTGC-AGCATCTAGAAAGCTT ACATGCATCCTAG-3' (SEQ ID NO: 5). The left region was isolated from pKOS20-56.1 as a 1.2 kb *EcoR* I - *Bgl* II fragment and cloned into the *EcoR* I - *BamH* I sites of pFDNEO-S (4), to give pKOS20-68. The right region was isolated from pKOS20-56.1 as a 1.2 kb *Hind* III - *Nsi* I fragment and cloned into the same sites of pKOS7-150 to give pKOS20-61.1. The 2.2 kb *BamH* I - *Hind* III fragment of pKOS20-68 was moved into the same sites of pKOS20-61.1 to give pKOS20-70, in which the neomycin resistance gene was inserted between the *rapQONML* flanking sequences.

Please amend paragraph [0139] starting on page 45 as follows:

I fragment and ligated to the *Bam*H I - *Pst* I sites of KC515 DNA and the DNA transfected into *S. lividans* TK24 protoplasts as described (6). Recombinant phage plaques were identified using PCR. Phage harboring the replacement cassette was mixed with freshly germinated spores of ATCC 29253 and plated onto modified oatmeal agar (10<sup>7</sup> - 10<sup>8</sup> spores per plate; MOI of 5-10) as described (9). After 20 h at 30°C, plates were overlayed with neomycin (10 μg/ml final concentration) and incubated about 10 days. Selected colonies were transferred to minimal medium containing 20 μg/ml neomycin and those that grew well after about 10 days were streaked on minimal medium containing 20 μg/ml neomycin to obtain pure clones. Clones were tested for the double crossover

event by patching to medium with neomycin alone and neomycin plus thiostrepton. Selected NeoR, ThioS strains were grown in Difco tryptic soy broth supplemented with 1% glucose, 100 mM MES (2-(N-morpholino)-ethane sulfonic acid) buffer, pH 6.0 and DNA was isolated as described (6). The DNA was analyzed by PCR for the presence of amplimers diagnostic of the double crossover replacement event. The primer pairs annealed to regions outside the left homologous sequence

## (5'-CGGGCGTCTGATCGACCAGGATGAGATGGG-3') (SEQ ID NO: 6)

and within the neo cassette promoter

## (5'-TATGTTGGTGTCATTCTACCAGAATCGGCAAAAGATGTCA-3') (SEQ ID NO: 7),

or outside the right homologous sequence (5'-GCGAGGGCGTAGCCCCGGCG-3') (SEQ ID NO: 8) and in the polylinker at the 3' end of the neo cassette

(5'-GTCGACCTGCAGGCATGCAAGCTT-3') (SEQ ID NO: 9). A strain in which the *rapQONML* region was correctly replaced by the *neo* cassette was designated KOS20-001.

## **AMENDMENTS TO THE DRAWINGS**

Please add new Figure 2 (appended herewith).